National Veterinary Services Laboratories

Testing Protocol

Real-Time RT-PCR for Detection of Virulent Newcastle Disease Virus in Clinical Samples

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Real-Time RT-PCR Test for Detection of Virulent Newcastle Disease Virus

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Real-Time RT-PCR Test for Detection of Virulent Newcastle Disease Virus

1. Introduction

1.1 Background

Virulent Newcastle disease (vNDV) is an acute, highly contagious and fatal disease of chickens of all ages. The disease also affects turkeys and numerous species of wild and captive birds. Virulent Newcastle disease is caused by avian paramyxovirus-1 (APMV-1) of the family *Paramyxoviridae,* genus *Avulavirus*. Acute vNDV in susceptible chickens can cause sudden death and high mortality with few or no clinical signs. Surviving birds may show respiratory distress, diarrhea, depression and central nervous system signs. The clinical signs of vNDV may resemble those of other diseases. Therefore, the signs are not considered pathognomonic. Virulent Newcastle disease is reportable to the World Organization for Animal Health (OIE). The causative virus must meet one of the following criteria for virulence: a virus that has an intracerebral pathogenicity index (ICPI) in day-old chicks of 0.7 or greater or presence of multiple basic amino acids at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of F1 protein.

The real-time reverse transcriptase-polymerase chain reaction (RRT-PCR) assay was developed to aid in the rapid diagnosis of avian paramyxovirus-1 infections in poultry. The technique utilizes a one step protocol with specific primers designed to amplify a portion of the genome that contains a target PCR sequence. Non-extendible fluorogenic hydrolysis/Taqman probes monitor the target PCR product formation at each cycle during the PCR reaction. The probes are labeled at the 5′ end with a reporter dye (e.g. FAM) and a quencher dye (e.g. blackhole quencher [BHQ-1]) at the 3′ end. The proximally located quencher dye absorbs the emission of the reporter dye as long as the probe is intact and not hybridized to the target. When the probe is hybridized to the target, the 5′ nuclease activity of Taq-polymerase will cause hydrolysis of the probe, separating the quencher from the reporter dye. This separation results in an increase in fluorescence emission of the reporter dye, which is detected spectrophotometrically and recorded. The amount of fluorescence recorded is proportional to the amount of target template in the samples.

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Real-Time RT-PCR Test for Detection of Virulent Newcastle Disease Virus

The APMV-1 (matrix) assay described in this protocol will detect viral RNA from both virulent and avirulent APMV-1 strains. Due to the lower diagnostic sensitivity of the CalMex assay (the fusion gene primer set described in AVPRO1505.01), new primer combinations were developed for the detection of vNDV viral RNA. This assay will be referred to as the vNDV assay and the primer set is the Creelan/CalMex primers. A description of the equivalency study for the validation of the Creelan/CalMex primers has been included in appendix 1. The vNDV (fusion gene) assay will detect most vNDV strains, including the one that infected U.S. poultry in CA, AZ and NV in 2002-03. The matrix assay respectively has a diagnostic sensitivity and specificity of 96.7% and 97.3% for the detection of APMV-1 RNA from chicken cloacal and tracheal swabs and 97.8% and 95.6%, for the detection of vNDV in chicken tissues. The vNDV assay respectively has a diagnostic sensitivity and specificity of 91.26% and 97.5%, for the detection of vNDV viral RNA from chicken cloacal and tracheal swabs.

Tissues from more than one bird should not be pooled together. However, tracheal swabs from up to 5 birds from one premises can be pooled together in 2-3.5 ml of brain heart infusion (BHI) broth, but should not be pooled with cloacal swabs. Tracheal swabs are the preferred specimen for the isolation of RNA. Validation data has demonstrated a decreased efficiency in the isolation of RNA from cloacal swabs due to the heavy load of organic material. It should be emphasized that the RRT-PCR technique will detect viral nucleic acid from infectious as well as noninfectious virus. For this reason, the RRT-PCR would not be the test of choice to determine if infectious APMV-1 was present in environmental samples.

Any specimen from outside a USDA quarantine zone that is positive with the APMV-1 (matrix) assay, regardless of the vNDV (fusion) assay results, should be referred to the National Veterinary Services Laboratories for further testing and characterization. Virus isolation and characterization is required to officially diagnose a foreign animal disease (FAD).

Virulent NDV is a zoonotic agent and causes conjunctivitis in humans. Personnel handling clinical samples or live virus should take appropriate safety precautions to avoid accidental introduction of the virus into the eyes. Appropriate safety precautions should include wearing disposable gloves, laboratory coat, and safety glasses. Care should be taken not to rub or touch the eyes before removing gloves and washing hands with soap and water.

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The procedure described here is used in the Diagnostic Virology Laboratory (DVL) of the National Veterinary Services Laboratories (NVSL). The brands of equipment listed in the protocol are used in the DVL; however, comparable equipment may also be used. Laboratories using this protocol should follow quality assurance procedures as they pertain to equipment maintenance, receiving specimens, and recording/reporting results.

1.2 Keywords

Virulent Newcastle disease virus (vNDV), real-time reverse-transcriptase polymerase chain reaction (RRT-PCR), avian paramyxovirus-1 (APMV-1), room temperature (RT), biological safety cabinet (BSC).

1.3 Collaboration

This protocol was developed in cooperation with the Southeast Poultry Research Laboratory, Agriculture Research Services, USDA, Athens, GA, and the California Animal Health and Food Safety Laboratory, University of California, Davis, CA.

2. Materials

2.1 Facilities/equipment/instrumentation

2.1.1 Surveillance samples originating from outside a known vNDV quarantine zone can be processed in a biosafety level 2 (BSL-2) laboratories. However, samples originating from inside a known vNDV quarantine zone or considered suspect for vNDV, should be handled under increased biosecurity. This includes restricted access to where the clinical samples are being handled until the samples have been rendered non-infectious. Once a clinical sample has been treated with lysis buffer for RNA extraction, the sample can be moved to a less restrictive environment to complete the RNA extraction and RRT-PCR analysis. Once a sample is biologically amplified (isolated in cell culture or chicken embryo) and has been confirmed or is suspected of being a vNDV virus, the virus should be handled at a higher containment level, specifically biosafety level 3 or preferably biosafety level 3-ag.

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2.1.2 Class II, HEPA filtered biological safety cabinets (BSCs) with UV germicidal lights are required (preferably 3, minimum of 2) to maintain sample integrity during processing and testing. In addition, the Trizol® extraction procedure should be performed in a class II BSC that is connected to an external exhaust plenum to minimize exposure to organic chemical fumes.

2.1.3 Refrigerator (4 C ± 2°)

2.1.4 -20 C (± 3°) freezer (not frost-free)

2.1.5 -70 C (± 5°) freezer

2.1.6 Micro centrifuge (non-refrigerated [International Equipment Co., MicroMax, Needham Heights, MA] and refrigerated [Hermle, Z 360K, Germany] or [Sorval® Heraeus Biofuge Fesco, Germany])

2.1.7 Vortex mixer

2.1.8 Assorted test tubes and Eppendorf tube racks

2.1.9 An integrated DNA/RNA amplification and detection instrument system that has the capability to detect specific sequences using hybridization probes. Instrumentation should be capable of exciting and detecting fluorescein-based probes (450-495nm, 500-550 nm, 565-590nm, and 630-750nm ranges). The current protocol was developed using the Cepheid Smart Cycler [(Cepheid® Smart Cycler, P#SC2000N1-1, Sunnyvale, CA). However, most if not all of the commercially available real-time PCR machines can detect the fluorescein-based probes, and likely can be used with this test. Based on past experience of transferring protocols between different machines, changes in cycling times and probe concentrations are often required to get equivalent sensitivity. Therefore, optimization of the assay on alternative machines is required. This optimization data needs to demonstrate that the alternative machine provides comparable sensitivity and limit of detection to the assay described in the current protocol with the Smart Cycler® machine. This data should be available for review by APHIS.

2.1.10 25µl Smart Cycler® tubes (Catalog #900-0022

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or 900-0003, Cepheid® Smart Cycler, Sunnyvale, CA)
2.1.11 PCR reaction tube refrigerated tube holder and mini-microcentrifuge to spin tubes. Both items are supplied with the Smart Cycler® real-time PCR machine.

2.1.12 QiaVac® 24 vacuum manifold (Valencia, CA) and vacuum pump with a capacity of 18-20 liter/min. (Gast MFG Corp., St. Louis, MO). Use of insufficient vacuum pressure may reduce RNA yield and purity. The vacuum manifold system with vacuum pump is optional, but is a highly recommended product for processing large numbers of samples for RRT-PCR.

2.2 Reagents/supplies

2.2.1 Molecular biology grade RNase-free sterile distilled water

2.2.2 *In vitro* transcribed NDV matrix-fusion gene positive control RNA (supplied by the NVSL, Ames, IA)

2.2.3 *Ethanol, absolute, ACS grade or better (**Caution: irritant, flammable)**

2.2.4 *Isopropanol, 99+% pure ACS grade or better **(Caution: irritant, flammable)**

2.2.5 *Chloroform, 99+% pure **(Caution: toxic)**

2.2.6 *Trizol® LS reagent **(Caution: toxic in contact with skin and if swallowed; causes burns.)** (Cat. #15596 or 10296, Life Technologies, Grand Island, NY)

2.2.7 Qiagen® RNeasy Extraction Kit (Cat. # 74103 20 preps, #74104 50 preps or #74106 250 preps, Qiagen®, Valencia, CA).

2.2.8 One-Step RT-PCR (Cat. #210210 or 210212, Qiagen®, Valencia, CA). Many one-step RT-PCR kits are commercially available. However, the Qiagen system has been tested extensively with good results. Other kits can be considered for use, but a minimum level of equivalency testing is required before substituting any reagents in the approved protocol. Currently, the only alternative kit that has been tested that has comparable test results to the Qiagen system is the Superscript One-Step RT-PCR System with Platinum Taq

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DNA Polymerase (Cat #10928-034 or 10928-042, Invitrogen, Carlsbad, CA). Appropriate changes to optimize the protocol for use with alternative reagents or RT-PCR kits, including cycling parameters, must be supported by an equivalency evaluation as previously noted.

2.2.9 Hydrolysis probes and primers (oligonucleotides) (Table 1) for the detection of vNDV. Suggested probe sources: Biosearch Technologies (http://blackholequenchers.com) and Operon (http://oligos.qiagen.com/). Suggested primer sources: Integrated DNA Technologies, (http://www.idtdna.com/), or Operon (http://oligos.qiagen.com/). Other companies can be used to order both primers and probes. It is strongly recommended that the probes and primers be purified to a high level to reduce nonspecific reactions. Purification is typically performed with HPLC purification as the minimum.

2.2.10 Ambion MagMAX®-96 Viral RNA Isolation Kit (Cat. # 1835-plate kit or 1929 for single tube kit) (Ambion®, Austin, TX 78744-1832)

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Table 1. NDV real-time RT-PCR probe and primer sequences.

Specificity and target gene	Primer/probe name-Genomic target	Sequence
APMV-1 (matrix)	M+4100** (forward primer)	5'-AGTGATGTGCTCGGACCTTC-3'
	M+4169** (matrix probe)	5'-[FAM]TTCTCTAGCAGTGGGACAGCCTGC[BHQ]-3'
	M-4220** (reverse primer)	5'-CCTGAGGAGAGGCATTTGCTA-3'
vNDV (fusion)*	F+4829** (forward primer)	5'-GGTGAGTCTATCCGGARGATACAAG-3'
	F+4894** Probe-1 (VFP-1) (virulent fusion)	5'-[FAM]AAGCGTTTCTGTCTCCTTCCTCCA[BHQ]-3'
	F-4939** (reverse primer)	5'-AGCTGTTGCAACCCCAAG-3'

*Validation testing with the vNDV primers and VFP-1 probe set has demonstrated that these reagents will detect the vast majority of mesogenic and velogenic APMV-1 viruses.

**Refers to the nucleotide position where the 5' end of the probe or primer anneals to the NDV genome. The FAM/TAMRA hybridizing probes were validated with the Smart Cycler I system. When using the Smart Cycler II system it is recommended that all hybridizing probes be labeled with FAM as a reporter dye and quenched with either Dabcyl or Black Hole™ quencher I. The Smart Cycler II system is not calibrated to use the TAMRA dye as a quencher. A TAMRA dye is read as background noise in channel 2 for the Smart Cycler II.

2.2.11 RNase Inhibitor, 40 units/µl (Promega, catalog #N2511 or N2515, Madison, WI)

2.2.12 $MgCl_2$, 25 mM (Promega, catalog #A3511 or A3513, Madison, WI)

2.2.13 TE buffer pH 8.3 (Promega #V6231 or V6232)

2.2.14 Qiagen® RNeasy Mini Kit (Qiagen #74104 or 74106)

2.2.15 14.3 M *β-mercaptoethanol (β-ME) (Sigma, M 6250) **Caution: toxic – dispense in a fume hood, wear gloves**

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2.2.16 Sterile, aerosol-resistant pipette tips of various sizes (1.0ml, 200-50µl, 100-10µl, 10-0.5µl)
2.2.17 1.7 ml microcentrifuge tubes (sterile)

2.2.18 Either powder-free latex or nitrile gloves may be used for any procedure requiring gloves

2.2.19 Calibrated pipettors from 0.5 µl to 1000 µl, 2 sets, one extra pipettor for DNA transfer

2.2.20 Ambion® Magnetic Stand-96 (Ambion Inc. catalog #10027, Austin, TX or an O-ring 96 well Magnetic-Ring Stand (Cat.# 10050) (Ambion, Austin, TX). Product #10027 has 96 ring magnets that pellet beads in a donut shape. Product #10050 has 24 big magnetic rods that pellet beads to one side of the wells.

2.2.21 Orbital shaker for 96 well plates (Lab-Line Titer Plate Shaker Model #4625, Melrose Park, ILL)

2.2.22 5-200 µl 12 channel pipetting tool (Matrix Technologies Corp., catalog #6012, Lowell, MA)

*Use caution when using these reagents. Refer to the individual Material Safety Data Sheet (MSDS) before handling any of these reagents.

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3 Preparation for the test

For this procedure, it is critical to have separate preparation areas and equipment for nucleic acid extraction, "clean" procedures, and work with amplified nucleic acid. The "clean" area is used for preparing reagents for use in the PCR procedure. Amplified c-DNA or sample RNA should never be introduced into this area. One biological safety cabinet should be designated for "clean" work only. There should also be a separate set of "clean" pipettors and tips, RNase-free water, tubes for reagent preparation, racks, and ice container which are designated for "clean" use only and never leave the area. A -20 C freezer should also be designated as "clean" for storage of reagents. A second biological safety cabinet, set of pipettors, and other equipment and reagents should be used for extraction procedures. Ideally, a third biological safety cabinet should be used for transfer of RNA to amplification tubes. Latex or nitrile gloves, in particular, must be worn throughout the procedure and must be changed frequently. RNA is very labile and easily degraded by RNases that are ubiquitous, including on human skin. Gloves also help protect the reagents and samples from other contaminating agents and cross-contamination that can adversely affect results. Always change gloves after working with sample RNA or amplified DNA. Always wear fresh gloves when working with "clean" reagents. Protective eyewear, gloves, and lab coats should be worn as some of the reagents used are toxic. It is recommended that the Trizol extraction procedure is conducted in a vented class II BSC.

3.1 Personnel qualifications/training

Personnel performing PCR procedures should be familiar with:

3.1.1 Preparation and proper handling of samples and reagents

3.1.2 Calibration, maintenance, and use of instruments included in this protocol

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3.2 Preparation of equipment/instrumentation

Refrigeration equipment, incubators, centrifuges, pipettors and thermal cyclers are calibrated and certified according to the respective institution standard operating procedures.

3.3 Disinfectants

Several classes of disinfectants, e.g. iodophores, phenolics, quaternary ammonia compounds, 70% alcohol, 10% sodium hypochlorite, and peroxigen compounds, will inactivate APMV-1 by destroying the lipid envelope of the virus. However, of the disinfectants listed above, only sodium hypochlorite (bleach) and peroxygen compounds (Virkon-S) have been shown to degrade nucleic acid as well as destroy infectivity of APMV-1. This is important when selecting a disinfectant to rid surfaces of contaminating nucleic acids.

3.4 Preparation of reagents/control procedures

3.4.1 Oligonucleotide primers

Prepare primers in a "clean" hood (see 3.0). Always wear gloves when dispensing primers.

Dilute primers to 200 pmol/µl (200µM) in 1X TE for the stock dilution and 20 pmol/µl in RNase-free water for the working dilution. Aliquot primers in small volumes to avoid excessive freeze/thaw cycles. For short-term storage of primers (<1 week), 4 C is acceptable. For longer term storage, -20 C or colder is recommended. Store stock primer solutions at -20 C or -70 C. 26.8 pmol of the vNDV forward primer, 13.4 pmol of the vNDV reverse primer, and 10 pmol of the matrix forward and reverse primers are added per 25 µl reaction. Additionally it is recommended that the PCR primers be, at a minimum, HPLC purified.

3.4.2 Hydrolysis Probe

Prepare probe in a "clean" hood (see 3.0). Always wear gloves when working with hydrolysis probes. Hydrolysis probes are light sensitive and should be protected from exposure to direct light. Diluted probes should be stored in amber sterile RNase free microcentrifuge tubes or tubes wrapped with foil.

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Dilute probes to 120 pmol/µl (120µM) in 1X TE for the stock dilution and to 6 pmol/µl in RNase-free water for the working dilution. Aliquot probe in small volumes to avoid excessive freeze/thaw cycles. Store diluted probes at -20 C and stock primer solutions at -20 C or -70 C. Avoid excessive freezing and thaw cycles. Diluted probe should not be frozen/thawed more than 4 times. A total of 6 pmol of the probe is added per 25 µl reaction.

3.4.3 Handling and dilution of Primers and Probe

Lyophilized primers and probes must be centrifuged briefly to ensure that the DNA pellet is at the bottom of the tube before they are opened and reconstituted. TE buffer should be used for the initial reconstitution of lyophilized primers and probes. Quantitation information will be supplied for each oligonucleotide primer (oligo) by the manufacturer.

An example of calculation for oligo reconstitution:

You have 17786 pmol of oligo (will be on oligo information sheet from manufacturer).

Need 200pmol/µl for stock concentration.

Divide pmol of oligo by the pmol/µl needed or:

$$\frac{17786 \text{ pmol}}{200 \text{ pmol/µl}} = 88.9 \text{µl of 1X TE}$$

The calculation for the probe is the same, except divide the number of probe pmol by 120 pmol/µl. Mix gently by tapping the tube and allow the oligo to resuspend for about 10 minutes before use.

Working stocks of primers should be 20 pmol/µl (20µM) and working stocks of probes should be 6 pmol/µl. Dilute the primers 1:10 and dilute the probe 1:20 in nuclease free H_2O (do not use TE buffer) for the working stocks.

Additional information on fluorescent probe handling and storage can found at: www.operon.com and www.idtdna.com ; www.idahotech.com.

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3.4.4 Prepare a 70% ethanol solution using 100% absolute ethanol and RNase-free water.

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3.4.5 Prepare 80% ethanol solution using 100% absolute ethanol and RNase-free water.

3.4.6 β-Mercaptoethanol (β-ME) must be added to the RNeasy RLT lysis buffer before use. Add 10µl β-ME per 1 ml RLT buffer. Buffer RLT is stable for 1 month after addition of β-ME. Be sure to date buffer after adding β-ME.

3.4.7 100% absolute ethanol is added to the RNeasy RPE wash buffer according to the Qiagen® kit directions.

3.4.8 The template for the positive controls is *in vitro* transcribed RNA from the NDV matrix and fusion protein gene. (200 ADV, NVSL, 1800 Dayton, Ave., Ames, IA)

3.4.9 Dilute RNase inhibitor to 13.3 units/µl with RNase free water.

3.4.10 The transcribed RNA positive control (provided by NVSL, Ames, IA) should be diluted to a working concentration that will consistently give a target C_t of approximately 25.0. Dilute the stock RNA to the suggested working dilution as described in the product insert sheet that will accompany the transcribed RNA positive control with sterile RNase free water. Run the diluted RNA on the RRT-PCR assay to determine if the positive control has a C_t that is within the acceptable range (section 6.2).

3.5 Preparation of samples

Perform all procedures with potentially live agents in an approved Class II biosafety cabinet with HEPA filtration. Always wear protective clothing and gloves when handling potentially infected tissues or live virus.

Samples received for testing are compared to accompanying paperwork to assure correct samples were received and that

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appropriate samples were submitted for the PCR test. Cases are logged into the logbook.

Pooled oropharyngeal/tracheal swabs (5 swabs/tube) are the specimens of choice for the PCR procedure and should be extracted using either the Qiagen® or Ambion® RNA extraction procedure. Appropriate tissue (spleen, lung, brain, intestine) should be processed by preparing a 10-20% tissue homogenate and extracting RNA using the Trizol® extraction procedure. Alternatively, $5mm^3$ piece tissues are added to 2.0 ml of brain heart infusion broth (BHI), frozen solid, thawed and centrifuged. The supernatant from this tissue pool (250µl) is extracted using the Trizol® procedure. Validation/equivalency data indicate that cloacal swab specimens are significantly less sensitive than oropharyngeal/tracheal swabs for the detection of APMV-1 by RRT-PCR. For this reason, cloacal swabs should be tested by virus isolation and not by RRT-PCR. **Tissues from more than one bird should not be pooled together.**

All samples should be processed in a Class II biological safety cabinet.

4 Performance of the test

Before beginning the RT-PCR test, place "clean" pipettors, racks, tips, etc. into the "clean" hood and expose to the UV germicidal light for several hours or overnight. Similarly, place the sample equipment into a separate hood and expose to the UV germicidal light.

4.1 Extraction of RNA from swab specimens (Qiagen® RNeasy method)

4.1.1 Vortex swab specimen fluid and transfer 500µl of sample into the microcentrifuge tube labeled with the specimen number.

4.1.2 Place 500 µl of Qiagen RLT with β-ME into the microcentrifuge tube. Vortex 15 sec. When processing a large number of specimens the RLT buffer can be mixed with the specimen by pipetting up and down vigorously 4 to 6 times.

4.1.3 Pulse spin to eliminate liquid specimen in the lid after vortexing. Add 500µl 70% ETOH and vortex

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well. Centrifuge lysed swab specimen for 5 min. at 5,000 X g in a microcentrifuge at RT.

4.1.4 Transfer all of the lysed specimen supernatant to a RNeasy® Qiagen column that has been marked to identify the specimen. Centrifuge for 15 sec. at ≥8,000 X g at RT*. Check to assure the entire specimen has flowed through the column. Repeat until all of specimen has been applied to the column.

*When working with a large quantity of specimens it is recommended to use a QiaVac® manifold to pull the specimen and wash solutions through the collection columns. This will increase the efficiency and eliminate the need to centrifuge the columns at the steps 4.1.3, 4.1.4, 4.1.5, 4.1.6 and 4.1.7.

4.1.5 Add 700µl of RW1 buffer to the RNeasy column and centrifuge for 15 sec. at ≥ 8,000 x g and place the column in a clean collection tube (the tube with RW1 flow through may be discarded).

4.1.6 Add 500µl RPE buffer to the RNeasy column and centrifuge for 15 sec. at ≥8,000 x g. Discard flow through from the collection tube.

4.1.7 Repeat for a total of 2 washes with RPE buffer discarding flow through from the collection tube. Following the last RPE wash place the RNeasy column in a new 2 ml collection tube.

4.1.8 Centrifuge the empty RNeasy column an extra 2 minutes at full speed and discard the collection tube.

4.1.9 Place the RNeasy column in an elution tube or a 1.5ml microfuge tube that has been marked with the specimen number and pipet 50µl RNase-free H_2O into the column. Do not touch the silica-gel membrane with the pipettor tip. Incubate at room temperature for 1 minute. Elute RNA by centrifuging for 1 minute at ≥10,000 rpm. Discard RNeasy column.

4.1.10 Store at 4 C until specimen is tested on RRT-PCR. RNA should be stored at 4 C for as short of period as possible before testing. If the sample cannot be tested within 24 hours, it should be stored at -20 C or colder.

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4.2 Trizol® LS Extraction for tissue samples

This procedure describes the extraction of total RNA from tissue using the Trizol® extraction reagent. The reagent is a mono-phasic solution of phenol and guanidine isothiocyanate. Addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase. The RNA is recovered from the aqueous phase by precipitation with isopropanol.

Different systems for RNA isolation are commercially available and potentially may work as well or better than the method described here. Alternative methods of RNA extraction can be substituted when comparison testing of new and standard methods show equivalency. This data should be available for review by APHIS.

Always wear protective clothing, eyewear, and gloves when working with extraction reagents.

4.2.1 Centrifuge tissue specimens (20% homogenates or tissue pools) at 1,500 x g for 30 min. Collect 250µl of the tissue supernatant and transfer to a 1.5 microcentrifuge tube. 750µl of Trizol® LS is added to the tube and sample is vortexed for 15 sec. Incubate at room temperature for 7 min.

4.2.2 Pulse spin to remove liquid from the tube lid.

4.2.3 Add 200µl 100% chloroform to the sample/Trizol homogenate. Vortex for 15 sec. Incubate at room temperature for 7 min.

4.2.4 Centrifuge at 12,000 x g for 15 min at room temperature.

4.2.5 Transfer 450µl of the upper aqueous layer to a separate microcentrifuge tube marked with sample number. **Caution: The transfer of organic phase material with the aqueous layer will inhibit the PCR reaction.** Add 500µl of 100% isopropanol. Invert tube several times to mix. Hold at room temperature for 10 min.

4.2.6 Centrifuge at 10,000 x g for 10 min at 4 C in the Hermle Z360K or Sorval Biofuge Fesco centrifuge.

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4.2.7 Decant liquid. Care should be taken to assure that the RNA pellet is not disturbed. Add 1.0 ml of 80% ethanol. Mix gently.

4.2.8 Centrifuge at 10,000 X g for 5 min at 4 C.

4.2.9 Decant ethanol. Invert tube on a clean tissue wipe and allow to air dry for 10 min. or until all visible signs of moisture are gone. It is important not to let the RNA pellet over-dry, as this will decrease its solubility.

4.2.10 Hydrate pellet in 50µl of RNase free water and allow to sit at 4 C for 1 hr to overnight. Briefly vortex to resuspend pellet before pipetting.

4.3. High throughput magnet bead RNA extraction (Ambion® MagMax method)

This procedure describes the extraction of total RNA from swab specimens using the Ambion® extraction reagent. The MagMAX® Viral RNA Isolation Kit is designed for rapid high throughput purification of total RNA from oropharyngeal/tracheal swab samples as well as cultured cells. The addition of guanidinium thiocyanate rapidly disrupts cellular membranes and inactivates cellular nucleases. Paramagnetic beads with a nucleic acid binding surface are added to the lysate to bind nucleic acids. The beads, containing the RNA, are then captured on magnets and the supernatant containing cell debris and other contaminants is removed during the wash procedures.

Different systems for RNA isolation are commercially available and potentially may work as well as the described procedure. Alternative methods can be substituted when comparison testing of new and standard methods show equivalency. This data should be available for review by APHIS.

4.3.1 Preparation of Ambion® Viral Isolation Kit Components

Lysis/Binding Solution. Sub aliquot the lysis/binding solution from the Ambion viral isolation kit into two different nuclease free containers. See table below to determine the quantity of lysis/binding solution needed. Set one aliquot aside for preparation of wash

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solution I mix. Add 1.0 µl Poly (A) RNA (kit provided) per 50 µl lysis/binding solution to the second aliquot and mix briefly. Following the addition of Poly (A) RNA add an equal amount (50 µl) of 100 % isopropanol to lysis/binding Poly (A) mixture to give a total volume of 101 µl per sample. Vortex well. **Lysis/binding solution is stable at room temperature for one month.** It is not recommended to store the prepared Viral Lysis/Binding Solution at 4° C or below as this may cause the carrier RNA to precipitate; if the solution is inadvertently stored at 4° C, warm it at 37° and shake to dissolve any precipitates before use.

Add carrier RNA to Lysis Solution and mix well before adding isopropanol, or carrier RNA may be very difficult to disperse.

1. Combine the following:	**1 Plate**	**4 Plates**
Viral Lysis/Binding Soln. Concentrate	6.25 ml	25.0 ml
Carrier RNA	125 µl	500 µl
2. Mix briefly, then add:		
100 % Isopropanol	6.25 ml	25.0 ml
3. Mix well by vortexing		

4.3.1.1 Bead Resuspension Mix. Dilute the Bead Resuspension Solution with Nuclease-free Water according to the table below. **Be sure to add 100% isopropanol to the mixture last or beads may clump together and be more difficult to enter into solution.** Store at RT.

Bead Resuspension Mix	Volume Per Well	Volume Per Plate
1. Bead Resuspension Solution	6.0 µl	750 µl
2. Nuclease-free Water	4.0 µl	500 µl
Mix briefly then add		
3. RNA Binding Beads	4.0 µl	500 µl
Mix briefly then add		
4. 100% Isopropanol	6.0 µl	750 µl
Mix well by vortexing Total Volume of Lysis/Binding Solution	20 µl	2500 µl

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4.3.1.2 Wash Solution I Mix. Mix the second sub-aliquot of lysis/binding solution in equal parts with 100% isopropanol. A total volume of 100 µl is used per sample. Store at RT.

4.3.1.3 Wash Solution II Mix. Mix 40 µl of Wash Solution II Concentrate with 160 µl of 100 % ethanol to gain a total volume of 200 µl per sample. Store at RT.

4.3.2 Procedure for Extraction of RNA

4.3.2.1 Vortex swab specimen and transfer 50 µl of sample into the corresponding well on the 96 well processing plate (supplied with kit).

4.3.2.2 Place 101 µl of viral lysis/binding solution with Poly (A) RNA to each well containing sample. After addition of the lysis/binding solution, the processing plate may be removed from the BSC. All remaining steps can be performed on the deck at room temperature. Shake plate on the plate shaker at moderate speed for 30 sec.

4.3.2.3 Add 20 µl of beads/binding mix to each well. Shake plate on orbital shaker for 4 minutes at approximately 550-600 rpm.

4.3.2.4 Capture/pellet the RNA Binding Beads on a magnetic stand. Pellet the beads on the Ambion Magnetic Stand-96 for 2 minutes and remove supernatant from beads (with plate still on magnet). Discard the supernatant. The mixture should become transparent, indicating the capture is complete.

4.3.2.5 Remove the plate from the magnetic stand.

4.3.2.6 Add 100 µl Wash Solution I Mix (with isopropanol added) to each well. Shake for 30 seconds at approximately 550-660 rpm. The RNA Binding Beads may not fully disperse during the step; this is expected, and it will not affect RNA purity or yield.

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4.3.2.7 Pellet the beads for 1 minute and remove supernatant as in step 4.2.2.4.

4.3.2.8 Add 100 µl Wash Solution II Mix (with ethanol added) to each well. Shake for 30 seconds at approximately 550-600 rpm.

4.3.2.9 Pellet the beads for 30 seconds and remove supernatant. Discard the supernatant. Remove the processing plate from the magnetic stand.

4.3.2.10 Repeat steps 4.2.2.8-4.2.2.9 to wash a second time with Wash Solution II.

4.3.2.11 Shake vigorously for 2 minutes (shaker dial position 9 Lab Line) to briefly dry the beads. It is important to remove residual ethanol from the samples. Residual ethanol may affect RT-PCR efficiency.

4.3.2.12 Add 50 µl of Elution Solution (RT) and shake for Four minutes at approximately 1000 rpm.

4.3.2.13 Pellet the beads for 2 minutes and transfer the RNA into sample tube, storage plate (kit provided), or RRT-PCR amplification plate.

4.3.2.14 Store at 4 C until specimen is tested on RRT-PCR. RNA should be stored at 4 C for as short of period as possible before testing. If the sample cannot be tested within 24 hours, it should be stored at -70 C.

4.4. Reverse-transcription and PCR

Two work areas are required for this procedure: a "clean" area with a dedicated BSC, freezer and supplies, and a thermal cycling area. Never introduce RNA/DNA material into the "clean" area and always change gloves before entering the "clean" area.

4.4.1 In the "clean" hood, prepare a master mix of the following reagents sufficient for the number of

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samples being tested. The amount given in the table is per sample.

This procedure was designed for the Cephied® Smart-Cycler (Cepheid®, Sunnyvale, CA).

Information on setting-up and programming the Smart Cycler can be found in the Smart Cycler user's manual. The conditions for the RT-PCR on the Smart Cycler are shown in tables 2 and 3.

Table 2. RT step thermocycling for Qiagen® one-step RT-PCR Kit.

RT Step	1 cycle	30 min.	50° C
		15 min.	95° C

Table 3. Thermocycling conditions for gene specific probe and primer sets.

Probe/Primer set		**Step**	**Time**	**Temp**
APMV-1 matrix	40 cycles	denaturation	10 sec.	94° C
		annealing	30 sec.	56° C
		extension	10 sec.	72° C
vNDV/VFP-1	40 cycles	denaturation	10 sec.	95° C
		annealing	30 sec.	58° C
		extension	10 sec.	72° C

Note: The fluorescence is acquired at the annealing step.

4.4.2 The real-time RT-PCR reaction should be prepared with the following components and volumes using the appropriate primer and probe set and cycling conditions. Set-up the reactions with the reaction tubes in the cooling block and use aerosol resistant pipet-tips.

Prepare the reaction mix (everything but the template) by pipetting: H_2O, kit supplied 5X reaction buffer, kit supplied dNTP's, forward and reverse primers, and 25mM $MgCl_2$ into a nuclease free microcentrifuge tube using the volumes per reaction for each reagent given in

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table 4. Next add the RNase inhibitor and enzyme. Add the probe last. Mix reagents and centrifuge briefly. Once the probe has been added minimize exposure of the reaction mix to light.

4.4.3 Add the reaction mix (17µl) to the Smart Cycler tubes (add the mix to the bottom of the cup at the top of the reaction tube).

4.4.4 Add 8 µl of purified test sample RNA to the Smart Cycler reaction tube using a pipettor designated for RNA transfer. Close the lid of the tube and number the reaction tubes according to test worksheet. Transfer 8µl of diluted transcribed RNA (200 ADV - provided by NVSL, Ames, IA) into the positive control reaction tube and 8µl of clean RNase free water into negative control reaction tube.

4.4.5 Centrifuge reaction tubes to enable the RNA reaction mixture to occupy the reaction portion of the tubes and to remove any air bubbles from the reading window of the PCR tubes.

4.4.6 Insert reaction tubes into thermal cycler and select the designated PCR run protocol, start run and enter sample identification as well as positive and negative control information into the sample identification portion of the results table. Save run.

Table 4a. Real-time RT-PCR reaction mix volumes and conditions for NDV with APMV-1 (matrix) primer/probe sets.

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Real-Time RT-PCR Test for Detection of Virulent Newcastle Disease Virus

	Volume Per Reaction	Final Concentration
H_2O	6.45 µl	
5X buffer	5	1X
25mM $MgCl_2$	1.25	3.75 mM*
dNTP's (10 mM each)	0.8	320 µM ea. dNTP
Forward Primer (20 pmol/ul)	0.5	10 pmol/25µl
Reverse Primer (20 pmol/ul)	0.5	10 pmol/25µl
Rnase Inhibitor (13.3 units/µl)	0.5	0.266 units/µl
Enzyme Mix	1.0	
Probe (6pmol/ul)	1.0	0.24 µM
MM per rxn	17	
Template	8	
Total	25µl	

*Qiagen buffer already contains 2.5 mM $MgCl_2$ at 1X concentration

Table 4b. Real-time RT-PCR reaction mix volumes and conditions for NDV with vNDV primer/probe set

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Real-Time RT-PCR Test for Detection of Virulent Newcastle Disease Virus

	Volume Per Reaction	Final Concentration
H_2O	5.69 µl	
5X buffer	5	1X
25mM $MgCl_2$	1.25	3.75 mM*
dNTP's (10 mM each)	0.8	320 µM ea. dNTP
Forward Primer (20 pmol/ul)	1.34	26.8 pmol/25µl
Reverse Primer (20 pmol/ul)	0.67	13.4 pmol/25µl
Rnase Inhibitor (13.3)units/µl	0.5	0.266 units/µl
Enzyme Mix	1.0	
Probe(6pmol/ul)	0.75	0.18 µM
MM per rxn	17	
Template	8	
Total	25µl	

*Qiagen buffer already contains 2.5 mM $MgCl_2$ at 1X concentration

5 Data Analysis Settings for the Cepheid® Smart Cycler

The Smart Cycler software provides multiple methods for determining the cycle threshold (C_t). All data analysis options discussed below are selected from the **Analysis Settings** from the **Views** screen. Changes in the standard default settings have been made to customize the analysis for the detection of vNDV with the matrix and vNDV primer/probe sets. Edit the Smart Cycler default analysis settings as describe below so the raw data will be analyzed according to the analysis parameters described.

Curve Analysis – Accept the "**Primary curve**" setting – The C_t is detected and reported at the cycle where the primary curve crosses the threshold.

Usage – Accept the default setting "**Assay**" for the FAM channel.

Background Subtraction – Accept the default value of "**ON**"
Background Minimum – accept the default value of "**5**" (Min). The Smart Cycler calculates the background of the fluorescence signal within the range of cycles defined by the Background Min Cycle value and the Background Max Cycle value for each site to measure the background noise.

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Background maximum cycle – Enter "**28"** as the maximum cycle for the calculation of the standard deviation of the fluorescence signal. The default is 40.

Threshold Settings – accept the default of "**Manual".**

Manual Threshold Fluorescence units – Enter "**25"** fluorescent units. It is critical that the C_t be above the background fluorescence. The closer the threshold is set to the background fluorescence the more sensitive the detection limit. However, if the threshold is set too close to the background fluorescence, background noise could cross the threshold and be reported incorrectly as a positive sample. By lowering the threshold fluorescence units from 30 to 25 the analytic sensitivity of the assay is increased. The possibility of reporting a false negative is reduced while the possibility of detecting a false positive is increased.

Boxcar Average – accept the default of "**0"**

The only changes from the Cepheid® Smart Cycler default were the Max background and threshold settings. With these analysis settings a specimen will be called positive (crosses the C_t) when the fluorescence units exceeds 25 units. These settings were designed to optimize the discrimination of positive and negative specimens for this particular protocol, but the results from each run still need to be verified by the user. The curve should be in the log-linear phase when crossing the threshold. By lowering the Max background, the incidence of traces with a "V" shape are reduced. For samples that still have a "V" shaped trace, the Max background setting can be incrementally lowered to 15 until the early cycles in the trace are approximately horizontal and aligned with zero fluorescence (Figures 2a and 2b). When lowering the Max background keep in mind that you want as many cycles as possible used to calculate the background subtraction. This is a correction on how the software handles the data and it decreases the number of cycles used to calculate the background and correct the curve. If you have questions about the trace, look at the trace with the background subtraction off. This shows the raw fluorescence data, and can sometimes aid interpretation.

The fluorescence trace of each specimen should be reviewed before accepting the Smart Cycler positive/negative result.

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Any specimen whose fluorescence trace has a gradual increase in fluorescence units and approaches, but does not cross the C_t, should be sent to the NVSL for further testing. These specimens are referred to as "late risers" (LS) and on some occasions may indicate a sample near the threshold of detection. At this time the significance of "late risers" samples is not known and testing by virus isolation is recommended.

6 Analysis of test results

6.1 The matrix APMV-1 assay is designed to detect APMV-1 including vaccine/lentogenic strains as well as vNDV (mesogenic/velogenic strains). The vNDV assay was designed specifically for the California 2002 and Mexico 2000 velogenic vNDV, but it has been shown to detect most mesogenic/velogenic strains of NDV. Because the matrix APMV-1 test is more sensitive (96.7%) than the vNDV test (91.26%), the matrix APMV-1 test is used to screen all samples. For APMV-1 positive samples, the vNDV assay is used to confirm the matrix test results. If both tests are positive, it provides strong evidence that vNDV is present in the sample. In some cases where the sample is at the limit of detection, a vNDV specimen can test positive with the APMV-1 (matrix) test and negative by the vNDV assay. Samples positive by the matrix primer/probe set that cannot be confirmed with the vNDV primer/probe set should be considered as suspect and additional samples collected for further testing. All suspect specimens should be tested by virus isolation. It is also possible that APMV-1 (matrix) positive, vNDV negative tests result from lentogenic or vaccine strain NDV isolates being in the sample. For samples that have a low cycle threshold (C_t of <30), where lower C_t means a stronger positive, confirmation that the APMV-1 (matrix) positive, vNDV negative samples are vaccine or lentogenic isolates can be made using different primer and probe sets. These primers and probes sets are still being optimized and will be included in future versions of this document.

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All samples positive with either RRT-PCR test should be confirmed by virus isolation. For samples outside a quarantine zone, these samples must be sent to the NVSL, since the NVSL is the reference laboratory for vNDV. Confirmation by virus isolation is required before an FAD will be "officially" diagnosed.

Table 5. Interpretation of the RRT-PCR results using the matrix and vNDV primer and probe sets

Matrix results	vNDV Results	vNDV RRT-PCR Interpretation
Positive	Positive	Positive
Positive	Negative	Suspect
Negative	Positive	Suspect

Any questionable samples should be re-tested. If results of the second test are unsatisfactory additional sampling from the flock or premises should be considered if possible.

6.2 Expected C_t for the transcribed RNA positive control

The transcribed RNA positive control should be diluted to a working concentration that will consistently give a target C_t of approximately 25.0. Any test run where the positive control has a C_t higher than 29.0 should be repeated to assure that test reproducibility is maintained. If the C_t of the positive control consistently runs lower than 20.0, recalibration/dilution of the positive control is suggested. Any time the positive control or any other growth curve C_t is lower than a 14.0 the background subtraction can be skewed.

6.3 Recommendations for evaluating fluorograms

Evaluation of the fluorogram with the following conditions may be helpful in determining results manually:

6.3.1. Identification of weak positives.

Remove all reactions with greater than 100 units increase in fluoresce from the graph (this changes the scale, making it easier to identify weak positives). (Figures 1a and 1b).

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Figure 1a. Example of a fluorogram from samples run on the Smart Cycler. All samples shown. Background subtraction is on. All analysis criteria are set to the customized default values. Note that scale is from 0 to 1000 fluorescence units (Y axis), making it difficult to evaluate weak positive samples.

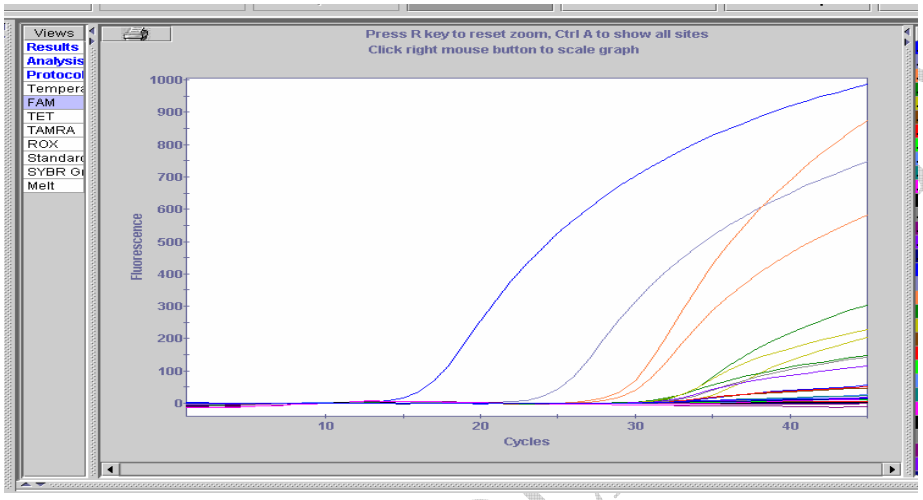
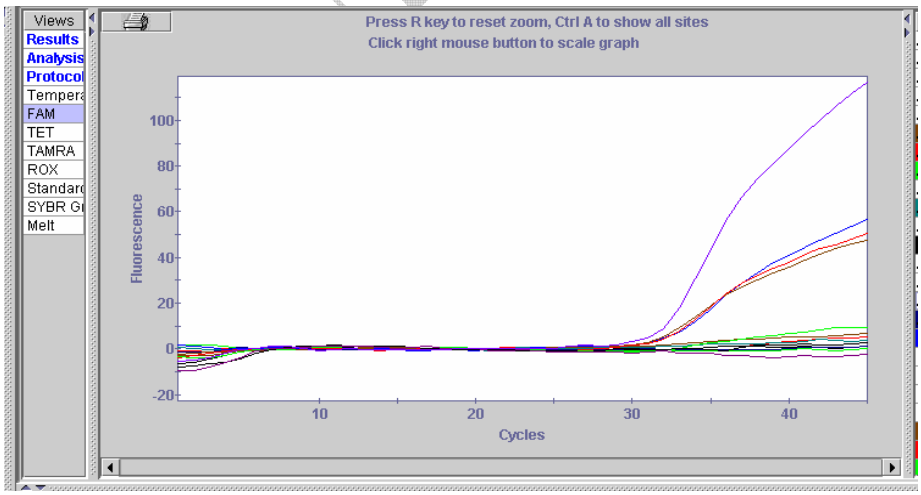


Figure 1b. Same fluorogram as figure 1a, however all samples which increased greater than 100units in fluorescence were removed from the graph. Note that the scale is from 0 to 120 fluorescence units (Y axis) making it easier to recognize weak positives.



6.3.2 If there are samples which have a "V" shaped fluorescence trace, incrementally lower the "background maximum cycles" (analysis settings table) by 5 cycles until the early cycles are approximately horizontal and aligned with zero fluorescence (Figures 2a and 2b).

Figure 2a. Example of a "V" shaped fluorescence trace. The background maximum cycle is set to the default of 40 (circled). All other analysis criteria are set to the default values. The negative control is shown for reference (horizontal line at zero).

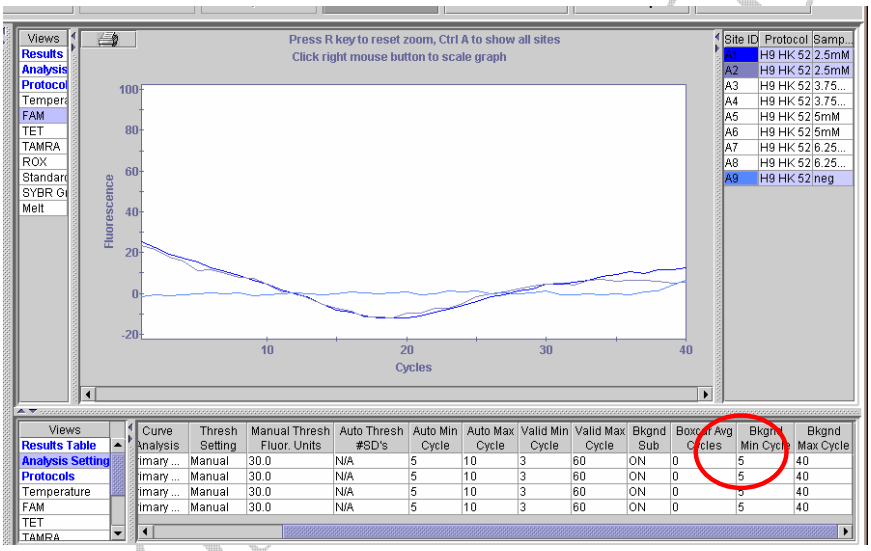


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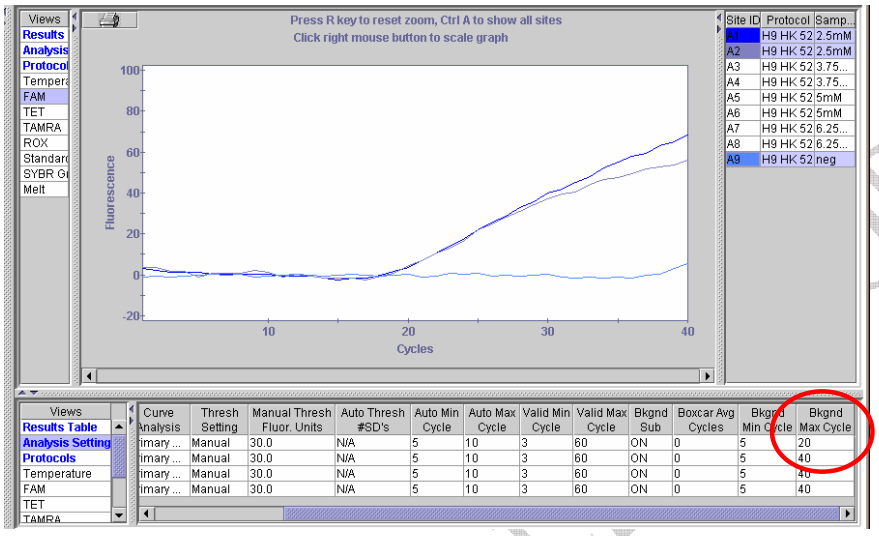
Figure 2b. Same fluorogram as figure 2a, however the background maximum cycles have been reduced to 20 (circled) to align the background fluorescence at 0 units. All other analysis criteria are set to the default values. The negative control is shown for reference (horizontal line at zero).


Press R key to reset zoom, Ctrl A to show all sites
Click right mouse button to scale graph
Fluorescence
Cycles
Site ID Protocol Samp...
A1 H9 HK 52 2.5mM
A2 H9 HK 52 2.5mM
A3 H9 HK 52 3.75...
A4 H9 HK 52 3.75...
A5 H9 HK 52 5mM
A6 H9 HK 52 5mM
A7 H9 HK 52 6.25...
A8 H9 HK 52 6.25...
A9 H9 HK 52 neg
Curve Analysis | Thresh Setting | Manual Thresh Fluor. Units | Auto Thresh #SD's | Auto Min Cycle | Auto Max Cycle | Valid Min Cycle | Valid Max Cycle | Bkgnd Sub | Boxcar Avg Cycles | Bkgnd Min Cycle | Bkgnd Max Cycle
imary ... Manual 30.0 N/A 5 10 3 60 ON 0 5 20
imary ... Manual 30.0 N/A 5 10 3 60 ON 0 5 40
imary ... Manual 30.0 N/A 5 10 3 60 ON 0 5 40
imary ... Manual 30.0 N/A 5 10 3 60 ON 0 5 40

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7 References

7.1 Aldous, E. W., M. S. Collins, A. McGoldrick, D.J. Alexander, Rapid pathotyping of Newcastle disease virus (NDV) using fluorogenic probes in a PCR assay, Veterinary Microbiology, 80, 2001. pp. 201-21.

7.2 Office of International des Epizooties Manual of Standards for Diagnostic Tests and Vaccines, Fourth edition 2000. pp. 221-222.

7.3 Spackman, E., Senne, D.A., Myers, T.J. et al. 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J. Clin. Micro. 40:3256-3260.

7.4 Wise, M.G., D.L. Suarez, B.S. Seal, J.C. Pedersen, D.A. Senne, D.J. King, D.R. Kapczynski, E. Spackman, Development of a Real-Time Reverse-Transcription PCR for Detection of Newcastle Disease Virus RNA in Clinical Samples. J. Clin. Micro. 2004. 42:329-338.

7.5 U.S. Department of Health and Human Services Public Health Service, Centers for Disease Control and Prevention and National Institutes of Health, Manual of Biosafety in Microbiological and Biomedical Laboratories, Fourth edition 1999.

8 Appendix 1

A temporal equivalency validation for the Creelan/CalMex assay was conducted with experimentally inoculated chickens. The equivalency validation included both acutely infected and pre-acute infected birds. Both tracheal and cloacal swabs were collected commencing on day one. The original APMV-1 matrix and CalMex RRT-PCR validation was conducted with field specimens collected during the 2002-03 CA vNDV outbreak. The original CalMex assay was shown to have a diagnostic sensitivity of 56.02% with acute and pre-actute specimens. This is significantly different from the original diagnostic sensitivity of 92.9% using field specimens from acutely infected birds. Due to the decreased sensitivity of the original CalMex assay, any specimen testing positive with the CalMex primer/probe was a strong positive with either the matrix or the Creelan/CalMex (vNDV) RRT-PCR assays. Diagnostic sensitivity and specificity data

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from both validation studies have been included for further illustration.

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Real-Time RT-PCR Test for Detection of Virulent Newcastle Disease Virus

vNDV RRT-PCR for Creelan/CalMex

Cree/Cal Mex Primers	+ Virus Isolation	Neg Virus Isolation
Pos RRT-PCR	303	4
Neg RRT-PCR	29	157

Diagnostic Specificity = 97.5%
Diagnostic Sensitivity = 91.26%

CalMex RRT-PCR Primer Set Using vNDV Equivalency Data

CalMex Primers	+ Virus Isolation	Neg Virus Isolation
Pos RRT-PCR	186	1
Neg RRT-PCR	146	160

Diagnostic Specificity = 99.37%
Diagnostic Sensitivity = 56.02%

CalMex RRT-PCR Validation Using Field Data

CalMex Primers	+ Virus Isolation	Neg Virus Isolation
Pos RRT-PCR	171	11
Neg RRT-PCR	13	1266

Diagnostic Specificity = 99.1%
Diagnostic Sensitivity = 92.9%

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9 Summary of Revisions

9.1 The concentration of Rnase Inhibitor in Table 4a and 4b was corrected. Version 1505.02 listed the use dilution of Rnase Inhibitor as 6.65 units/µl in Table 4a and 6.65 units/50µl in Table 4b. The final concentration of Rnase Inhibitor was listed as 0.266 units in both Table 4a and 4b. The correct use dilution of Rnase Inhibitor is 13.3 units/µl. The correct final concentration is 0.266 units/µl.

9.2 Section 2.2.17 was corrected to include either powder-free latex or nitrile gloves for any procedure requiring gloves. Neither latex nor nitrile gloves were specified for any of the specific procedures.

9.3 The title of the protocol was changed from Real-Time RT-PCR for Detection of Exotic Newcastle Disease Virus in Clinical Samples to Real-Time RT-PCR for Detection of Virulent Newcastle Disease Virus in Clinical Samples.

9.4 Exotic Newcastle disease (END) was replaced by virulent Newcastle disease virus (vNDV) throughout the body of the protocol.

9.5 Version 03, February 24, 2005, supercedes version 02, September 29, 2004.

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